# Genomic rearrangements of the *CDKN2A* locus are infrequent in Italian malignant melanoma families without evidence of *CDKN2A/CDK4* point mutations

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**Predisposition to familial cutaneous malignant melanoma has been associated with mutations in the *CDKN2A* and *CDK4* genes. However, only a small subgroup of melanoma pedigrees harbour *CDKN2A* or *CDK4* germline mutations. It is possible that other types of *CDKN2A* rearrangements, not detectable by routine PCR-based approaches, are involved in a fraction of melanoma cases negative for point sequence changes. In order to gain insights on the possible role of *CDKN2A* large deletions or duplications in melanoma susceptibility in the Italian population, we screened a series of 124 cutaneous malignant melanoma families referred to five national medical/cancer genetics centres. All probands were negative for point mutations in *CDKN2A* and *CDK4*. All samples were tested by MLPA (multiplex ligation-dependent probe amplification), and the results were confirmed by real-time quantitative PCR in a subset of 53 cases. No genomic rearrangements were detected in this series, one of the largest so far investigated. These data suggest that large deletions/duplications in the *CDKN2A* locus are infrequently involved in the development of familial melanoma in the Italian population. Based on these results, routine search for these rearrangements in *CDKN2A*- and *CDK4*-mutation negative melanoma families is not warranted, although it would be reasonable to pursue it in selected cases with very strong family history and/or showing linkage to 9p21. *Melanoma Res* 18:431–437 © 2008 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

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## Introduction

Familial cutaneous malignant melanoma (CMM) represents 5–10% of all CMM cases. Genetic linkage analyses of CMM kindreds have identified the *CDKN2A* gene, located on human chromosome band 9p21, as the major melanoma susceptibility gene. *CDKN2A* encodes two cell cycle regulatory proteins, p16INK4a and p14ARF; the former is transcribed by exons 1α, 2, and 3, while the latter utilizes an alternative first exon, *CDKN2A* exon 1β, coupled with exons 2 and 3 in a different reading frame. p16INK4a is a cyclin-dependent kinase inhibitor reducing the level of phosphorylation of RB1 through inhibition of cyclinD/cdk4 complexes, whereas p14ARF acts as a p53 activator through its ability to inhibit MDM2-mediated p53 degradation [1].

So far *CDKN2A* is the major gene linked to hereditary malignant melanoma. Only a few families have been reported with germline mutations in the *CDK4* gene, encoding cyclin dependent kinase 4 [2–4]. Germline *CDKN2A* mutations have been identified in 20–40% of melanoma-prone families [5–11]. The fractions of cases positive for *CDKN2A* mutations among familial CMM patients vary by geographic region, with higher frequencies occurring in regions of low incidence [11–13].

Most *CDKN2A* germline mutations identified to date are located within the coding sequence. Mutations in non coding regions, such as deep intronic regions and the promoter/5′ UTR [14–17], and rare mutations in exon 1β have also been described, but they only account for a small subset of all 9p21-linked families [18,19]. Indeed, it is currently difficult to estimate how many families are 9p21-linked, given the difficulty of verifying segregation in small families.

Methods used to screen for *CDKN2A* mutations are usually based on traditional PCR amplification and

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sequencing of individual exons. Large deletions or rearrangements, undetectable by conventional methods, may underlie some familial cases where no *CDKN2A* mutations are found. To date, a systematic search for *CDKN2A* genomic rearrangements has been performed in only a few studies and very few constitutional deletions involving the whole or a portion of the *CDKN2A* locus have been described [20–26].

The frequency of *CDKN2A* heterozygotes is relatively high in Italy, probably owing to the spread of common founder mutations [27–29]. To assess the role of *CDKN2A* large quantitative alterations in the Italian population and to establish whether screening for large rearrangements should be included in the national Italian protocol for genetic testing for hereditary melanoma (*http://www.sigu.net/download.php?view.87*), we screened 124 Italian melanoma families without detectable *CDKN2A* and *CDK4* point mutations. In order to maximize sensitivity and specificity, we used two distinct approaches: multiplex ligation-dependent probe amplification (MLPA) [30], that enables analysis of a region encompassing the *CDKN2A* locus and further flanking genes, and real-time quantitative PCR.

## Materials and methods

### Patients and samples

The study included 118 families referred to five medical genetics or cancer genetics centres in Florence, Genoa, Milan (European Institute of Oncology, IEO, and Fondazione IRCCS Istituto Nazionale dei Tumori, INT), and Padua until July 2007. Six additional families included in the study were from IMI (Italian Melanoma Intergroup) centers (Aviano, Turin, Pisa, and Naples) and had been referred to Genoa for counseling and testing. Overall, 124 unrelated probands were enrolled in this study. Written informed consent to genetic investigations, approved by the local ethics committees, was obtained from each patient.

Forty-two kindreds have been previously reported to be negative for *CDKN2A/CDK4* point mutations [10,11,29,31], while the remaining 82 families have never been described.

Families were considered to be eligible when $\geq 2$ CMM cases were present, and were divided into the following groups: (a) families with more than three affected members ($n = 24$), (b) families with two first-degree relatives affected ($n = 80$), (c) families with two second-degree relatives affected ($n = 13$), (d) families with two third-degree relatives affected ($n = 7$). All diagnoses were confirmed by viewing histopathology reports, clinical records or death certificates.

Mutation testing had been performed by direct sequencing in accordance with the national protocol for clinical genetic testing for hereditary melanoma (*http://www.sigu.net/download.php?view.87*), which provides for screening for point mutations within the coding sequence and intron/exon borders of *CDKN2A* (exons 1β, 1α, 2, and 3) and of *CDK4* exon 2. Families from Florence, Genoa, Milan INT and Padua had been tested locally, while those recruited by Milan IEO had been tested in Genoa. None had detectable mutations.

A control population comprised 96 lymphocyte DNA samples from healthy individuals without any history of cancer among first-degree relatives was used to assess the frequency of a 6 bp deletion identified within the promoter region.

DNA derived from the Jurkat cell line (New England Biolabs, Ipswich, MA) was chosen as a reference sample for dosage analysis since this cell line carries a homozygous deletion of the *CDKN2A* locus [32]. In order to artificially reproduce a hemideleted sample, DNAs derived from the Jurkat cell line and from one of the above-mentioned control samples were mixed in 1 : 1 proportions.

Genomic DNA was prepared from peripheral blood leukocytes or lymphoblastoid cell lines obtained from melanoma-affected probands by phenol/chloroform extraction or using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

### Multiplex ligation-dependent probe amplification analysis

Multiplex ligation-dependent probe amplification analysis was performed in Florence for probands from all families, except those recruited in Padua, who underwent MLPA analysis locally, using the same protocol. The *CDKN2A* locus was analysed using the 9p21 MLPA kit (P024B) (MRC-Holland, Amsterdam, Netherlands) according to the manufacturer's instructions. This probemix contains nine different probes for *CDKN2A* (five located in the four coding exons and four located in the promoter regions of either $CDKN2A^{ARF}$ or $CDKN2A^{INK4A}$), 15 probes for *CDKN2B*, *MTAP* and other surrounding genes in the 9p21 region, and 15 control probes located on different chromosomes.

MLPA fragments were visualized on an ABI310 Automated Capillary DNA Sequencer (Applied Biosystems, Foster City, CA), using ABI POP-4 polymer, and GeneScan-TAMRA 500 size standard (Applied Biosystems). Data were analysed with the ABI PRISM GeneScan software (Applied Biosystems) and gene dosage quotients were calculated using Coffalyser MLPA DAT Software (MRC Holland, *www.mrc-holland.com/pages/indexpag.html*). This software uses normalization against 15 autosomal control probes, considering peak areas as the quantitative measure of DNA content.

For each *CDKN2A* fragment analyzed, samples were considered as wild-type (with two alleles), hemideleted (with one allele) or with a biallelic deletion when their quotients were 0.70–1.30, close to 0.5, and 0, respectively, as suggested by the manufacturer (*www.mrc-holland.com/pages/coffalyser_pagepag.html*).

### Real time PCR analysis

Real Time quantitative PCR was performed for *CDKN2A* exons 1β, 1α, 2, 3 on a Rotor-Gene 6000 Instrument (Corbett Research, Sidney, Australia) using SYBR Green as intercalating fluorescent dye. The *NF2* gene, located on chromosome 22q12, was used as an internal reference locus.

Lymphocyte DNAs from 10 control subjects were used as reference normal samples for the optimization of experimental conditions, DNA derived from the Jurkat cell line was used as a homozygous deleted sample and the 1 : 1 mixture of Jurkat and wild-type DNA was used as a hemideleted sample.

All primer sequences (Table 1) were designed using the Primer 3 program [33]. One µl of sample DNA (10 ng) was added to the PCR reaction mixture containing 2X SYBR Green Master Mix and 200 nmol/l forward and reverse primers in a final volume of 10 µl. All analyses were performed in triplicate. The PCR amplification profile was as follows: initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s, annealing for 40 s and extension at 72°C for 20 s. Detection of the fluorescent product was carried out during the annealing period.

Data analysis was performed using the comparative Delta Delta Ct (ΔΔCt) method [34]. The relative gene copy number was calculated by the expression $2^{-\Delta\Delta Ct}$; in order to establish the range of values for each group, standard deviations (SD) from the mean value were also calculated.

### Sequencing of the *CDKN2A* promoter region

A 522 bp fragment within the $CDKN2A^{ARF}$ promoter region (nt −923_−1444; Genbank accession no. NM_058195), containing the hybridization site for an MLPA probe, was analysed by direct sequencing, using the following amplification primers: forward 5′-GTCC GAGTTCCTGGACGAG-3′ and reverse 5′-AGCACCGA GTCCTTTGTGTC-3′.

**Table 1 Primers and conditions used for quantitative real-time PCR**

| Exon | Primer | Sequence | Annealing temperature (°C) | PCR product (bp) |
|---|---|---|---|---|
| Ex 1β | P14 1BF | GTTAAGGGGGCAGGAGTG | 56 | 171 |
| | P14 1BR | GGGATGTGAACCACGAAA | | |
| Ex 1α | P16 1AF | GGTCGGGTAGAGGAGGTG | 56 | 157 |
| | P16 1AR | CAATTCCCCTGCAAACT | | |
| Ex 2 | P16 2F | CTTCCTGGACACGCTGGT | 60 | 161 |
| | P16 2R | CATGGTTACTGCCTCTGGTG | | |
| Ex 3 | P16 3F | AAGTATTTCAATGCCGGTAGG | 56 | 177 |
| | P16 3R | GGACCTTCGGTGACTGATGA | | |

## Results

### Multiplex ligation-dependent probe amplification analysis

Preliminary experiments conducted on three control samples, on the Jurkat cell line and on a 1 : 1 mixture reproducing a monoallelic deletion of the full *CDNK2A* locus yielded results in line with the expected gene dosage, thus demonstrating the reliability of the MLPA approach (Fig. 1).

In five out of 124 familial melanoma samples, values (0.47–0.57) in the range corresponding to a condition of hemizygous deletion were detected for the peak corresponding to the promoter region upstream of exon 1β.

All samples were shown to contain two copies of other *CDKN2A* fragments and of surrounding regions investigated by the kit.

### Real-time gene dosage

In order to assess the accuracy of real-time quantitative PCR to reveal dosage differences at the *CDKN2A* locus, we first tested DNA from 10 control samples, from the Jurkat cell line and from one artificial hemizygous sample. These results were useful to define the ranges of values corresponding to wild type, hemideleted and homodeleted samples (Table 2). The results of real-time gene dosage confirmed the absence of large deletions in the *CDKN2A* coding regions in this familial CMM series.

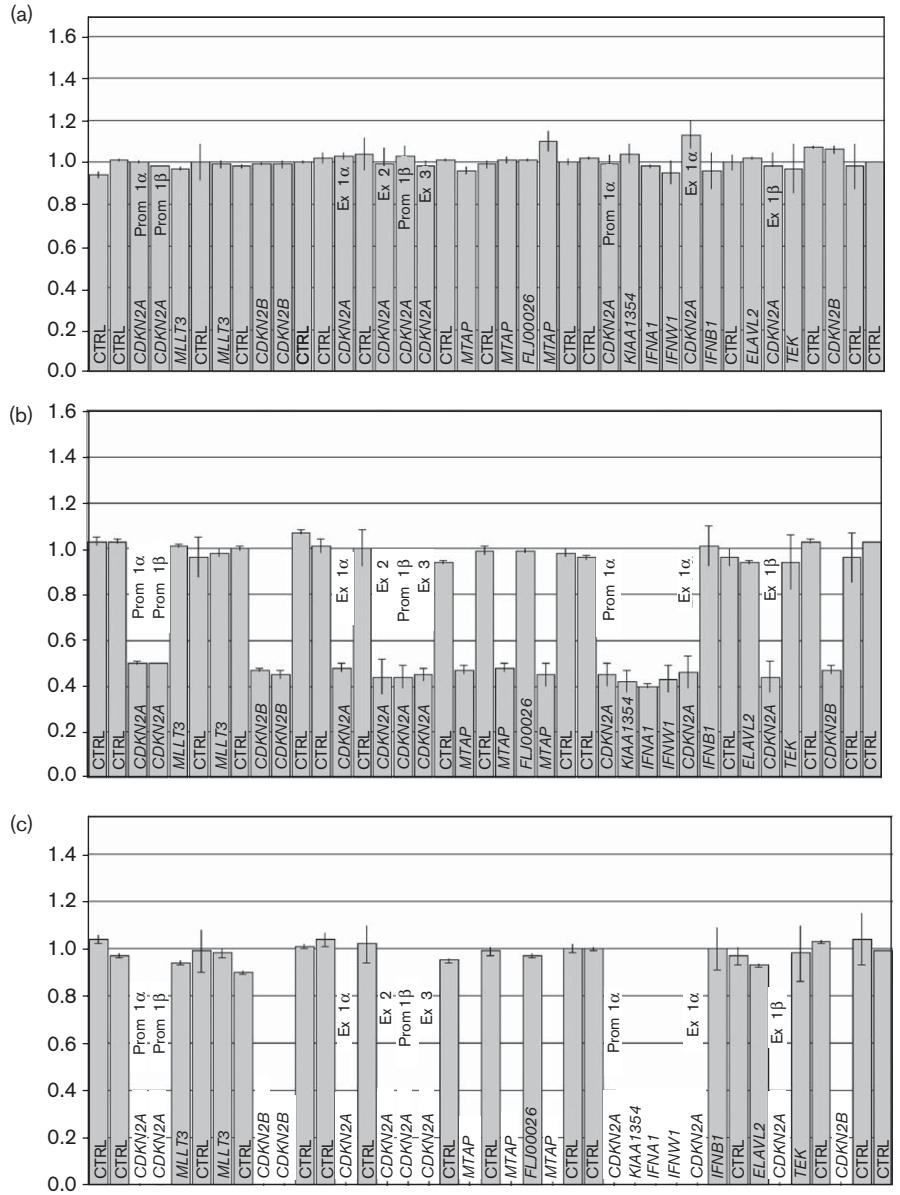
### Sequencing of the CDKN2A promoter region

In order to verify whether the reduction in the peak area corresponding to the $CDKN2A^{ARF}$ promoter region observed by MLPA in five samples was due to a genomic rearrangement or to variations within the sequence recognized by the MLPA probe, we performed direct sequencing of a 522 bp tract of the region upstream of exon 1β in these samples. A 6 bp deletion (c.−1173_−1178del) located within the site of the probe used for hybridization was detected in all five samples. Segregation analysis was undertaken in a CMM pedigree: the proband's unaffected father, considered as an obligate carrier since his brother (the proband's uncle) was affected, did not have the variant identified in his affected son. The 6 bp deletion was also observed in a heterozygous state in three of 96 control samples.

### Estimated frequency of deletions in Italian melanoma kindreds

Since no deletion events were detected in this series of 248 *CDKN2A* alleles, the proportion of *CDKN2A* deletions in Italian CMM families can be estimated between 0 and 1.2% at 95% confidence level.

**Fig. 1**




MLPA analysis of the *CDKN2A* region. (a) Wild-type control sample. (b) Hemideleted sample (Jurkat:control 1 : 1 mixture). (c) Jurkat DNA (with homozygous deletion). Symbols of investigated loci are shown on each bar (CTRL: control locus).

**Table 2 Range of values for Real Time gene dosage analysis**

| Sample | No. of *CDKN2A* copies | Range | $2^{-\Delta\Delta Ct}$ Values | | | |
|---|---|---|---|---|---|---|
| | | | Exon 1β | Exon 1α | Exon 2 | Exon 3 |
| Controls | 2 | Average | 1,01 | 1,02 | 0,94 | 1,02 |
| | | +2SD | 1,20 | 1,33 | 1,22 | 1,39 |
| | | −2SD | 0,82 | 0,71 | 0,66 | 0,65 |
| Jurkat | 0 | Average | 0,00 | 0,00 | 0,00 | 0,00 |
| Jurkat : Control 1 : 1 mixture | 1 | Average | 0,51 | 0,46 | 0,42 | 0,48 |
| | | +2SD | 0,63 | 0,44 | 0,44 | 0,53 |
| | | −2SD | 0,39 | 0,48 | 0,40 | 0,43 |

SD: Standard deviation.

## Discussion

Although *CDKN2A* is considered as the major melanoma susceptibility gene, mutations of this gene have been identified in only 20–40% of melanoma-prone families and 8–16% of patients with sporadic multiple primary cutaneous melanomas [5–11,35–37]. This finding suggests either the involvement of other genes or the existence of *CDKN2A* mutations not detectable by traditional PCR and sequencing of individual exons. The presence of large deletions or other genomic rearrangements in 9p21, that were not investigated in

earlier studies, might explain susceptibility in melanoma families in whom mutations at this locus have not been identified to date.

A few constitutional deletions involving either portions of or the whole *CDKN2A* locus have been described in single-case studies. Bauhau *et al.* [20] identified a large germline deletion ablating the whole p16/p14/p15 gene cluster and a smaller deletion involving p14 in two melanoma-neural system tumour (NST) families. A germline deletion of exon 1β has also been detected in a melanoma-neural tumour family by Randerson-Moor *et al.* [21]. The same exon 1β deletion was investigated with negative results in a series of 30 patients belonging to 24 Jewish melanoma-NST families, using microsatellite analysis and gene dosage with TaqMan probes [38]. More recently, a large deletion involving exon 1α and half of exon 2 has been identified in a Norwegian family by combined MLPA and transcript analysis [22].

A systematic search for *CDKN2A* genomic rearrangements in a large cohort of patients has been performed in only a few studies. Mistry *et al.* [24] analyzed the prevalence of 9p21 deletions in 93 UK melanoma families without any detectable *CDKN2A* or *CDK4* mutations. Three unrelated families were shown to harbour partial *CDKN2A* hemizygous deletions by MLPA analysis and the results were confirmed by semiquantitative PCR, microsatellite typing and sequencing. One of these rearrangements, previously reported by the same group [21], removed exon 1β and its promoter region and was found in two families, while the third family showed a novel $CDKN2A^{INK4A}$ deletion involving exons 1α, 2 and 3.

In a study performed using a combination of real-time quantitative PCR, linkage, and transcript analysis on 36 French familial CMM cases, a single 8474 bp germline deletion, ablating exon 1β, was identified in a family with five CMM affected members [25]. More recently, a study performed on a larger French series comprised of 167 patients identified a single 2935 bp deletion removing exon 2 by MLPA [26]. Finally, no *CDKN2A* alterations were observed in two other studies investigating for large genomic alterations, performed by real-time quantitative PCR in 23 familial CMM cases from France and by MLPA analysis on 16 Polish CMM families, respectively [9,23].

Worldwide studies suggest that the presence and frequency of *CDKN2A* mutations in familial melanoma can be different across continents and across groups in the same continent or even in the same country [11,13,29]. These observations prompted us to undertake a comprehensive analysis of constitutional deletions/duplications in one of the largest *CDKN2A* point mutation-negative familial CMM series so far investigated, in order to gain insights on the possible role of *CDKN2A* large rearrangements in melanoma susceptibility in the Italian population.

MLPA was used as the screening method for the whole series of 124 samples. MLPA is a rapid and effective technique that allows analysis of the whole region encompassing the *CDKN2A* locus and neighbouring 9p21 regions. However, occasionally it may yield false positives due to the presence of sequence variants located within the regions covered by the hybridization probes. Recently, a Norwegian study described the presence of a deletion with an exonic breakpoint located upstream of the MLPA probe hybridization site, that was therefore associated with a signal of normal intensity [22]. We therefore decided to confirm the normal MLPA results obtained on a subset of samples, for which sufficient DNA was available, by means of real-time quantitative PCR, using primers for different areas of the same exons.

No gross rearrangements in the *CDKN2A* coding regions and in the p16-specific promoter were detected in this large sample series. The absence of large deletions in the *CDKN2A* coding region was confirmed with both methods in 53 samples.

In five samples, MLPA analysis showed a reduction of the peak area corresponding to the promoter region of exon 1β. Direct sequencing revealed in all these cases the presence of a 6 bp deletion within the target sequence hybridizing to the MLPA probe. The same deletion was found in five probands and in three of 96 healthy controls, with allelic frequencies of 0.02 and 0.015 in the familial CMM and control population, respectively. In addition, it did not segregate with the disease phenotype in one melanoma family investigated. Therefore, this deletion represents a low frequency polymorphism probably not implicated in disease predisposition, although its involvement as a low-moderate risk factor for CMM cannot be completely ruled out. This issue would need investigation of larger familial CMM series and/or case-control studies.

Our study confirms that MLPA is a suitable method for large deletion analysis in familial CMM, as it allows the screening of a large genomic region, including areas of high sequence complexity and GC content, such as the *CDKN2A* locus, in a single PCR reaction. Simultaneous application of an independent method, real-time quantitative PCR, confirmed that alterations not detectable by MLPA should be quite rare.

We found that large rearrangements of the *CDKN2A* locus are infrequently involved in the development of familial CMM in the Italian population. These results, combined with previous data obtained in patients from other countries, are at variance with those obtained in other

cancer family syndromes, for which germline deletions of susceptibility genes (such as *BRCA1*, *MLH1*, *MSH2*, and *VHL*) have been shown to represent a frequent mutation mechanism underlying disease predisposition [39–41]. The low frequency of deletions in familial CMM could be related to specific features of the *CDKN2A* locus, such as its relatively small size, that makes it a less frequent mutation target, and, possibly, other structural characteristics.

In conclusion, since large deletions involving *CDKN2A* are rare, routine search for these rearrangements in *CDKN2A*- and *CDK4*-mutation negative CMM families does not seem to be warranted, although it would be reasonable to pursue it in selected cases with very strong family history and/or showing linkage to 9p21.

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